New Classical Conditioning Models of Drug Use in Mice

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Abstract

Conditioned Place Preference (CPP) is one of the models most frequently used to study drug use and addiction in animals such as mice and rats. CPP has a number of advantages, including low cost, ease of use and versatility. However, the model also has disadvantages: the novelty confound, difficulty in creating dose-effect curves, and inability to study discrete cues. It is important to determine whether alternate classical conditioning models can eliminate some of the limitations of CPP while maintaining its advantages. Here we did not find conditioned preference to odors. However, we were able to both demonstrate and later eliminate taste aversion. We found that mice did not spend more time investigating a scent after it had been repeatedly paired with morphine. Mice did develop an aversion for flavored food after it had been paired with morphine, which is consistent with previous research. Most importantly, when mice were given repeated injections of morphine to postpone withdrawal, they no longer developed an aversion for flavored food that was paired with morphine. Our results demonstrate that it may be possible to eliminate aversion and possibly even to create a preference for food paired with a drug, despite the fact that previous research has almost exclusively shown taste aversion. Demonstrating the practicability of eliminating taste aversion is the first step in establishing CTP as a viable model for drug abuse. Although more research is needed to further develop the model, CTP could serve as a useful complement or even replacement for CPP.

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Drug addiction is one of the most serious problems facing the United States today. Preliminary estimates suggest that there were 64,000 drug overdose deaths in 2016 (National Center for Health Statistics, 2017). This means that drug overdoses are now responsible for more deaths in the United States every year than AIDS at the peak of the HIV/AIDS crisis (Gawande, 2017). Drug overdoses are now the leading cause of death in Americans under the age of 50 and research suggests that overdose deaths are primarily responsible for the decrease in life expectancy that occurred from 2000 to 2015 in the United States (Dowell et al., 2017; Reynolds, 2015).

Opioids, both synthetic and non-synthetic, are the main driver of the increase in overdose deaths, accounting for over sixty percent. Fentanyl/fentanyl analogue overdoses alone increased 540% from 2013 to 2016 and now account for more deaths than heroin or prescription opioids (National Center for Health Statistics, 2017).

Although they receive far less attention, non-opioids are still responsible for almost two out of every five drug overdoses. Methamphetamine and cocaine overdoses were responsible for the deaths of over 18,000 people in 2016. Like opioid overdoses, non-opioid overdose deaths have been increasing for several years (National Center for Health Statistics, 2017).

For ethical and practical reasons, the majority of experimental research on drug addiction is performed using animal models (Merchant et al., 2013). Classical conditioning-based paradigms have become some of the most popular of these animal models because of their relatively low cost and ease of use (Bardo and Bevins, 2000; Prus et al., 2009). Classical conditioning models are based on the principle that if a neutral stimulus is repeatedly paired with

an emotionally salient stimulus, then the neutral stimulus will begin to take on the emotional salience of the second stimulus. Animals will then begin to respond to the "neutral" stimulus the way they would to the emotionally salient stimulus.

By far the most popular classical conditioning model used for drug addiction is Conditioned Place Preference (CPP). In CPP, a room is used as the neutral stimulus and a drug is used as the emotionally salient stimulus. After the two have been repeatedly paired, animals will spend more or less time in the drug-paired room depending on whether the specific drug acted as a positive or negative stimulus. The CPP model has several advantages, including its speed, ease of use, and ability to measure both rewarding and aversive stimuli (Bardo and Bevins, 2000). However, the model also has several limitations, such as the possibility of confounding due to withdrawal and the model's inability to study interactions between environmental (e.g. a place) and discrete (e.g. a specific object or odor) stimuli.

New classical conditioning models could retain the benefits of CPP, while possibly eliminating several of its limitations. These new models would operate similarly to CPP, but would use a discrete stimulus, such as an odor, taste or object, as the neutral stimulus. Although these models work based on the same principle as CPP, little to no research has been performed on them. It is important to study these models in order to determine whether they have the same potential uses and advantages as CPP and whether they can resolve some of the limitations inherent in the CPP model currently used in labs throughout the world.

This paper will briefly explore the history of classical conditioning models and the potential advantages and disadvantages of both CPP and alternate classical conditioning models based on discrete stimuli such as taste, odor or object recognition. The purpose of the present research is to determine whether mice can be classically conditioned to associate odor or taste

stimuli with morphine and to determine whether these stimuli are feasible candidates for future classical conditioning models of drug addiction.

Operant conditioning models mimic the process of drug addiction in the real world, but have several disadvantages

The oldest animal models for drug addiction are based on the principles of operant conditioning (Bardo and Bevins, 2000). In operant conditioning, an animal learns to associate a behavior with a specific consequence and increases or decreases the frequency of the behavior based on the consequence (Goodenough et al., 2007). For example, a dog that is given a treat whenever it rolls over will be more likely to roll over in the future.

One commonly used operant conditioning model is self-administration of the drug in question (see Table 1; Bardo and Bevins, 2000). During self-administration, subjects perform an action, such as press a lever, in order to receive a dose of a drug that is delivered through a surgically-implanted catheter (Panlilio and Goldberg, 2007). The major advantage of self-administration is that it closely mimics the way drug addiction occurs in the real world, in which the rewarding sensation caused by the drug acts as a reinforcer that leads to further drug use (Panlilio and Goldberg, 2007).

Nevertheless, self-administration has several limitations. It generally involves special equipment and surgery to insert the catheter and the procedure can be difficult, costly and time-consuming (Bardo and Bevins, 2000). The animal chooses how much of the drug to self-administer, so researchers have little control over the dose of the drug the animal receives. The fact that animals control the dose of drug they receive also means that self-administration produces inverted U-shaped dose-effect curves, which are difficult to analyze. Inverted U-shaped

curves are produced because animals tend to press less for both small doses of drug, which are not very rewarding, and for high doses of drug, which satisfy the animal so that it needs fewer injections of the drug. Finally, self-administration is only capable of measuring reward, although many drugs produce aversion in addition to or instead of reward.

Because of their relatively low cost and ease of use, classical conditioning paradigms have the potential to complement or even replace older operant conditioning models (Bardo and Bevins, 2000; Prus et al., 2009). Perhaps unsurprisingly, over the past two decades a type of classical conditioning known as Conditioned Place Preference (CPP) has become one of the most popular animal models for studying drug use (Tzschentke, 2007).

Conditioned Place Preference is based on the principles of classical conditioning

Classical conditioning was first described in the 1920s by Ivan Pavlov (Pavlov, 1927). Pavlov observed that when dogs were repeatedly given meat powder after the ringing of a bell, the dogs began to salivate at the sound of the bell, rather than only after the presentation of the meat itself (Pavlov, 1927).

In its simplest form, classical conditioning occurs when a previously neutral stimulus (the conditioned stimulus or CS) is repeatedly paired with a stimulus with some type of emotional salience (the unconditioned stimulus or US). Over time, behavior that was previously performed in response to the US (the unconditioned response or UR) begins to be performed in response to the CS alone (the conditioned response or CR) (Goodenough et al., 2009). For example, a rat may attempt to escape its cage (UR) in response to an electric shock (US). If the electric shock is repeatedly paired with a blue light (CS), the rat will begin attempting to escape in response to the blue light alone (CR).

The classical conditioning paradigm can also be applied to study drug addiction. Today, a form of classical conditioning known as Conditioned Place Preference (CPP) is widely used to study drug use and drug-seeking behavior. The first precursor to the modern CPP test is often credited to Spragg, who in 1940 found that chimpanzees that were addicted to morphine would choose a white box that contained a syringe with their daily morphine injection to a black box that contained a banana. Spragg's experiment used an object (the box) as the conditioned stimulus, but the paradigm was later adapted by Beach (1957), who used place as the conditioned stimulus instead. Beach discovered that both morphine-dependent and non-morphine-dependent rats preferred the white arm of a y-maze to the black arm after the white arm had been repeatedly paired with morphine.

The modern CPP apparatus consists of a box with two chambers connected by a doorway (Tzschentke, 1998). The chambers differ in some way, most commonly in the texture of the floor and/or the color of the walls, so that the animal can differentiate between them (Bardo and Bevins, 2000). In another common variant of the CPP experiment, the apparatus contains three chambers instead of two. The two conditioning chambers are connected by a third "neutral" chamber that the mouse is placed in to begin the exploration and test trials (Tzschentke, 2015).

During the first day, or "exploration phase", an animal, most commonly a mouse or a rat, is allowed to explore both chambers. During the conditioning phase, the doorway is blocked so the animal cannot move between the chambers. During each conditioning session, the animal is injected with either the drug of interest or saline. The drug of interest (US) is always paired with the same chamber (CS), while the saline is always paired with the other chamber as a control (Tzschentke, 1998).

After conditioning has finished, the animal is again allowed to explore both chambers. An animal that spends significantly greater time in the chamber that was paired with the drug is demonstrating a preference for the drug (Tzschentke, 2015). This preference is generally interpreted as showing drug-seeking behavior. In contrast, an animal that spends significantly greater time in the chamber that was paired with the saline is demonstrating avoidance of the drug (Tzschentke, 2015).

The current uses of CPP suggest that new conditioning models could be used in a wide variety of settings and experiments

CPP is the only classical conditioning model that is currently in widespread use in the field of drug addiction. CPP is used in a wide variety of settings and experiments. Although alternate classical conditioning models are not presently in use, understanding the scope of current CPP use can be used to help determine what some of the possible utilizations of new classical conditioning models could be. Because new classical conditioning models would be based on the same principle as CPP and would most likely operate similarly, they could likely be utilized in many, if not all, of the same areas in which CPP is currently used.

Although mice and rats are the most commonly used animals in CPP, conditioning has also been observed in other animals including zebrafish, squirrels, hamsters and chickens (Collier et al., 2014; Lahvis et al., 2015). Several different versions of the apparatus have also been developed, including one common version that has multiple chambers, allowing the effects of multiple drugs to be compared (Tzschentke, 1998).

One of the most well-established uses of CPP is to test the addictive or aversive properties of different drugs (Tzschentke, 1998). Dozens, if not hundreds, of drugs have been

tested using this method (Tzschentke, 1998). Animals show a reliable CPP for stimulants such as cocaine and amphetamines, opioids and nicotine, among other frequently abused drugs (Prus et al., 2009; Tzschentke, 1998). Although drugs are the most common focus of CPP research, CPP has also been shown in response to a wide array of naturally rewarding stimuli including food, novel stimuli, sweet liquids and sexual interaction (Bardo and Bevins, 2000; Tzschentke, 1998).

In addition to determining whether a drug is addictive, CPP can be used to understand the mechanisms through which it acts on the brain. By using agonists and antagonists that show specificity for one type of receptor, researchers can determine which receptors are responsible for the drug's effect (Tzschentke, 1998). These techniques have shown that drugs that support acquisition of CPP almost always act on the mesolimbic dopamine system and that the D2 receptor is primarily responsible for the rewarding effects caused by extracellular dopamine (Prus et al., 2009; Tzschentke, 1998).

By lesioning certain areas of the brain and then testing to see whether a drug still causes CPP, it is possible to determine whether that area is involved in the reward pathway that the drug triggers (Tzschentke, 1998). For example, lesions of the ventral striatum block amphetamine-induced CPP, suggesting that the ventral striatum is involved in the neuronal pathway that amphetamines act on (Olmstead and Franklin, 1996; Olmstead and Franklin, 1997)

In the past fifteen years, the increased availability of transgenic animals has greatly expanded the use of CPP in the study of individual differences in drug use and addiction (Tzschentke, 2007). In genetic studies, transgenic or knockout animals and control wild-type animals complete the same conditioning paradigm (Pandolfo et al., 2009). The two groups are then compared to determine whether more or less conditioning took place in the transgenic animals than in the wild type animals (Pandolfo et al., 2009). By knocking out genes or using

animal models for human diseases/disorders, it is possible to study whether specific genes, gender, disorders such as attention deficit hyperactivity disorder and other individual differences affect someone's potential of developing an addiction (Hilderbrand and Lasek, 2014; Pandolfo et al., 2009; Tzschentke, 1998).

CPP has many advantages that new classical conditioning models would share

CPP is now one of the most popular behavioral paradigms used to study drug effects and drug addiction in large part due to the model's many advantages, including its low cost, ease of use and ability to measure both reward and aversion (see Table 1; Tzschentke, 2007). Because any new classical conditioning models would be based on the same underlying principle as CPP and would probably have a similar protocol to CPP, new models would likely share most of these advantages.

One of the most important advantages that has contributed to CPP's widespread use is the model's low cost and ease of use. The materials are relatively cheap and easy to acquire and, because most versions of the experiment do not involve surgery, it requires little training to implement (Bardo and Bevins, 2000; Prus et al., 2009; Tzschentke, 1998). Analysis of CPP is also easy because it produces a monophasic, or S-shaped, dose-effect curve. Dose-effect curves show the effect of a drug at each dose and S-shaped curves are easier to analyze statistically than the inverted U-shaped dose-effect curves produced by self-administration (Bardo and Bevins, 2000).

Depending on the exact protocol, the CPP procedure can be completed in two weeks or less with only one conditioning session per day (Bardo and Bevins, 2000; Prus et al., 2009; Tzschentke, 1998). Conditioning can often be seen even after only one pairing of the drug and

chamber (Bardo and Bevins, 2000). The small number of drug/place pairings is convenient for the researcher and reduces or eliminates the occurrence of confounds from tolerance and sensitization (Bardo and Bevins, 2000). Self-administration, one of the only other widely-used techniques to study drug addiction, requires a much larger number of drug injections, which can lead to tolerance or sensitization to the drug, both of which may alter the results of the experiment (Bardo and Bevins, 2000). Additionally, self-administration requires animals to receive doses of the drug throughout the procedure. In contrast, in CPP animals are only injected with saline during the pretest and posttest. Because drugs often alter behavior while in the bloodstream, the fact that CPP allows animals to be tested in a drug-free state eliminates another confound (Bardo and Bevins, 2000).

Self-administration measures reward based on how many times an animal is willing to press a lever to receive a dose of a drug. Because lever-presses to receive a drug is self-administration's only measure, the model is not capable of measuring aversion. CPP is capable of measuring both reward and aversion based on whether animals increase (reward) or decrease (aversion) the amount of time they spend in the room paired with the drug (Bardo and Bevins, 2000). The ability to measure aversion is important because many drugs cause different effects at different doses or after different lengths of time using the drug and because drugs may cause aversive withdrawal effects, all of which are important to measure and understand (Brownell and Gold, 2012). Finally, several drugs, such as LSD and buspirone, cannot be self-administered by rodent models but can be studied using CPP (Prus et al., 2009).

CPP has several limitations, some of which would be solved by new classical conditioning models

Despite the advantages of using CPP, the method does suffer from several limitations (see Table 1). New classical conditioning models based on discrete stimuli have the potential to solve several of these limitations, while others would most likely be inherent in any classical conditioning-based model.

Disadvantages common to all classical conditioning models

The similarity between CPP and new classical conditioning models means that they will share many of the same advantages, but also means that they will likely share several of the same limitations. One of the largest disadvantages of CPP is its low face validity: the model bears little resemblance to the situations in which most humans use drugs (Prus et al., 2009). In CPP, as with possible new classical conditioning models, the subject has no control over whether they receive a drug. In contrast, in a natural environment the subject almost always chooses to take a drug (Mueller and de Wit, 2011). This difference may influence the neural pathways that are involved in the response to the drug (Mueller and de Wit, 2011). For example, concentrations of extracellular dopamine and acetylcholine in the nucleus accumbens, which have been linked to the reinforcing effects of several drugs, are higher in rats that self-administer cocaine than in rats on the same drug schedule who do not self-administer the drug (Hemby et al., 1997; Mark et al., 1999).

An additional limitation for CPP that would not be solved by newer models is the possibility of a novelty confound (Bardo and Bevins, 2000). Rats and other animals prefer novel stimuli and environments over known stimuli (Bardo and Bevins, 2000). If a drug blocks

memory formation, then animals may prefer the chamber paired with the drug not because they associate it with the drug, but because the chamber itself appears more novel to them than the other room. (Bardo and Bevins, 2000). This same effect would likely apply to any stimuli that were paired with the drug, including objects, odors and tastes. However, three-chamber CPP experiments have found that animals prefer a drug-paired chamber over a novel chamber, suggesting that most drugs do add at least some reward value in addition to any novelty effects (Bardo and Bevins, 2000).

Finally, the interpretation of CPP can be made difficult by the fact that most animals tend to prefer one chamber over the other before conditioning has even begun (Bardo and Bevins, 2000). This same preference would likely be seen with any stimuli that could potentially be paired with a drug. If the drug is paired with the chamber the animal initially prefers, ceiling effects may obscure any effect of the drug, but if the drug is paired with the non-preferred chamber, the results may overstate the amount of conditioning that occurred (Bardo and Bevins, 2000). In order to correct for this tendency, researchers must choose between an unbiased design, in which a standard randomization procedure is followed without regard to initial preference, and a biased design, in which the drug is paired with the preferred chamber for half the animals and the non-preferred chamber for the other half of the animals (Tzschentke, 1998).

Disadvantages that could be solved by new models

New classical conditioning models have the potential to solve several of CPP's limitations. The first of these disadvantages is that it is much more difficult to create dose-effect curves with CPP than with self-administration. Creating dose-effect curves with CPP is difficult because a new group of animals is needed to test every drug dose and the dose cannot be adjusted during the experiment based on observations about its effect (Bardo and Bevins, 2000).

Classical conditioning models based on a different type of stimulus could eliminate this problem by using multiple objects/odors/etc. and conditioning each stimulus to a different dose of the drug (Kennedy et al., 2016). For example, an animal could be injected with 15 mg/kg of morphine in the presence of a sphere, five mg/kg of morphine in the presence of a cube, and saline in the presence of a pyramid. During the testing phase, the animal would have access to all of the stimuli and the amounts of time that the animal spent with each stimulus would represent a different point on the dose-effect curve (Kennedy et al., 2016).

Another disadvantage of CPP is that differences in chamber preference between the pretest and posttest may not necessarily prove that the animal was demonstrating a preference for the drug-paired chamber. In most CPP apparatuses, excluding three-chamber designs, the animal is forced to choose between two chambers. If the animal spends more time in one chamber, it may not because it prefers that chamber, but because it is avoiding the other chamber (Kennedy et al., 2016). This is an especially important confound in drug research because, if the study is not timed correctly, the animal may be experiencing aversive withdrawal from the drug at the same time as it is placed in the saline-paired chamber. The animal may then come to associate the saline-paired chamber with withdrawal and spend more time in the drug-paired chamber, not because of any positive effect of the drug, but because it is avoiding the withdrawal-associated chamber (Olmstead and Burns, 2005).

Models using discrete stimuli, such as objects or odors, would almost certainly measure conditioning based on the amount of time that the animal spent investigating different stimuli (Kennedy et al., 2016). For example, if a mouse spent more time investigating a cotton swab with a drug-paired odor than a cotton-swab with a saline-paired odor. This would eliminate any confounding from aversion to the saline-paired stimulus because the animal would not be forced

to choose between the two. Instead, it could choose not to investigate either stimulus (Kennedy et al., 2016).

Finally, CPP is limited in the types of conditioning, both operant and classical, that it can be used to study. In contrast to CPP, which is based on the principles of classical conditioning, humans primarily develop drug preference through operant conditioning; humans learn to associate a behavior (taking a drug) with a rewarding stimulus (the effects caused by the drug) and so are more likely to repeat the behavior in the future (Prus et al., 2009).

Despite this difference, there is ample evidence that human drug users do experience classical conditioning as well as operant conditioning (O'Brien et al., 1992). Smoking paraphernalia, such as cigarettes or ashtrays, have been shown to increase drug cravings in human users (Conklin, 2006). Similarly to CPP, even environments that the smoker associates with smoking, such as a café or bar, can cause drug cravings (Conklin, 2006). Childs and de Wit (2009) performed a version of CPP in humans using d-amphetamine and found that subjects rated their liking of the amphetamine-paired room significantly higher than the placebo-paired room.

In order to better understand why people continue using drugs and how best to help them stop, it is important to continue studying the role of classical conditioning in drug use and how it interacts with other types of learning like operant conditioning (Kennedy et al., 2016). However, CPP is limited even on this front. Because it measures preference for one chamber vs. a different chamber, CPP is well designed to measure general environmental cues that drug users may come to associate with their drug. These environmental stimuli might include a bar, a house, or a room where someone uses a drug. Although general stimuli are important, discrete stimuli, including drug paraphernalia such as lighters or pill bottles, people that someone often uses drugs with or

odors and tastes that someone comes to associate with a drug are important as well. CPP is poorly equipped to answer questions about these discrete stimuli.

Although all classical conditioning models are limited in their ability to study operant condition, new classical conditioning models might provide a closer approximation to the discrete stimuli that drug users encounter. These models could be designed to specifically examine different types of discrete stimuli, including objects, odors or tastes that drug users may learn to associate with their drug. Expanding the number and type of conditioning models that are available could allow researchers to study many different types of drug cues with greater depth and specificity.

New classical conditioning models have the potential to complement CPP in drug addiction research

Despite the considerable amount of research that has been performed using the CPP model, almost no research has been performed on the viability of classical conditioning models that use other types of stimuli as the CS. Stimuli such as objects, odors or tastes could theoretically all be used to elicit a preference after being paired with a drug (See Table 1). These alternate conditioning models would likely have the same advantages as CPP and could solve several of CPPs limitations (Kennedy et al., 2016).

Conditioned Object Preference

Conditioned object preference (COP) is similar to CPP, but an object serves as the CS instead of a room (Kennedy et al., 2016). During conditioning, one object is repeatedly paired with a drug while a different object is repeatedly paired with saline. During the test trial, both

objects are placed in a cage with the animal and the amount of time that the animal spends investigating each object is measured (Kennedy et al., 2016).

Kennedy et al. (2016) performed the procedure in rats with promising results. Rats spent more time investigating an object paired with cocaine than an object paired with saline. When different objects were paired with different doses of cocaine, rats spent the most time investigating the high-dose-paired object, an intermediate amount of time investigating the low-dose-paired object, and the least amount of time investigating the saline-paired object, demonstrating that COP could be used to create dose-effect curves using only one group of animals (Kennedy et al., 2016). The rats also spent the majority of their time engaging in activities other than investigating the objects, supporting the hypothesis that the model would not be vulnerable to the forced-choice confound that is seen in CPP (Kennedy et al., 2016)

In another part of the study, rats were conditioned through repeated drug-object pairings in one environment. Afterward, the animals were switched to a new environment where the objects were repeatedly presented without the drug. The rats at first continued to show a preference for the cocaine-paired object, but soon exhibited extinction of the behavior. When the rats were returned to their original environment and were again presented with the objects in the absence of the drug, the rats' preference for the cocaine-paired object reemerged (Kennedy et al., 2016). The finding is significant for two reasons. First, it is consistent with classical conditioning theory and further validates the efficacy of the model. Second, it demonstrates that COP may have the potential to allow researchers to study how environmental stimuli modulate the response to discrete stimuli, which is another important area of drug research (Kennedy et al., 2016).

A second group repeated the COP experiment using mice instead of rats (Song and Gewirtz, unpublished data). This time, no significant results were found. The difference in the results between the two experiments may have been because rats naturally spend more time exploring and investigating their environment than mice (Stranahan, 2011). Because object investigation is not a normal part of mouse behavior, the mice may have treated the objects as an irrelevant part of their environment and not learned to associate the experimental object with cocaine. If stimuli were chosen that make up a more important part of the mouse's repertoire, mice might be successfully conditioned to associate the stimulus with a drug.

Conditioned Odor Preference

Odor is another stimulus that could potentially be used for drug conditioning. Although almost no studies have specifically examined conditioned odor preference as a model for drug use, many experiments have shown that it is possible to classically condition animals using odor as the CS. Research on invertebrates has shown that animals can associate odor with nutritional and reproductive stimuli. For example, honeybees learn to associate odors with sucrose water and respond to those odors by extending their proboscises (Menzel, 1999). Male crickets can be conditioned to associate an odor with a rival male cricket and respond to the presence of the odor by producing larger spermataphores for transfer to female crickets (Lyons, 2006).

In vertebrates, odor conditioning has been studied primarily in rats and mice because odor stimuli are especially salient to these species. Odor conditioning is also often used with newborns because of the difficulty of performing CPP with most young animals (Bouslama et al., 2005; Heyser et al., 1991). Newborn rats prefer odors that have been paired with infusions of milk and newborn mice prefer odors that have been paired with the rewarding stimulus of being gently stroked with a paintbrush (Johanson and Teicher, 1980). Most importantly, odor

conditioning has been shown in response to drug stimuli. One study found that newborn rats prefer odors associated with cocaine, which provides direct support for the plausibility of using odor preference as a model for drug use (Heyser et al., 1990).

Even human infants can learn to associate odors with a rewarding stroking stimulus, which suggests that odor conditioning may play an important role in human behavior (Sullivan et al. 1991). This is important because it supports the feasibility for odor preference as a model for studying specific discrete cues whose results could be generalizable to humans.

Although the majority of odor conditioning research has been performed in newborns, odor conditioning has also been demonstrated in adult mice (Goddyn et al., 2008; Svyf, 2014). Unlike object exploration, odors play an important role in many mouse behaviors. Mice use odors to find food, determine how closely related they are to other mice, choose appropriate mates, detect and avoid predators, and perform other essential behaviors (Apfelbach, 2005; Gilder and Slater, 1978; Yang and Crowley, 2009). Because odors are involved in so many important parts of a mouse's behavioral repertoire, mice may be more likely to attend to them and have a heightened ability to learn to associate them with paired stimuli such as drugs.

In fact, odor conditioning may be important in determining certain behaviors in adult mice in their natural environment. Early research showed that male rats learn through classical conditioning to produce ultrasonic vocalizations, which are an important part of courtship behavior, in response to the odor of female urine (Barbehenn, 1980; Bean, 1982). More recent research suggests that this response is at least partially instinctive, but has not completely eliminated the role of classical conditioning (Nyby et al., 1983).

In summary, odor conditioning has been shown in a variety of different species. Although no research has directly studied odor conditioning as a model for drug use in adult mice, research in newborn mice and rats and the importance of odor in the behavior of adult mice suggests that odor conditioning may be feasible in adult mice. Finally, the presence of odor preference in human infants suggests that any results from conditioned odor preference models may be at least partially generalizable to humans.

Conditioned Taste Preference

Tastes are in many ways unique as compared to any other classically conditioned stimuli. Conditioned Taste Aversion (CTA) was first observed in the 1950s and has since been the subject of a large body of research (Garcia et al., 1955; Logue, 1979). In a typical CTA study, an animal, usually a rat or a mouse, is given saccharine-flavored water (CS) to drink (Logue, 1979). Anywhere from several minutes to 24 hours later, the animal is given lithium chloride or a similar emetic agent (US) (Hunt and Amit, 1986; Logue, 1979). The animal becomes sick (UR) and, in future trials, will refuse to drink saccharine-flavored water (CR) (Hunt and Amit, 1986).

CTA is distinct from other forms of classical conditioning in several ways. First, in most types of classical conditioning, the US must be presented only seconds or minutes after the CS or the animal cannot learn to associate the two (Logue, 1979). In CTA, the US is usually presented an hour or more after the CS and learning can occur when up to 24 hours pass between presentation of the CS and the US (Hunt and Amit, 1986). Second, CTA occurs much more rapidly (normally after only one trial) than other forms of conditioning, which usually require multiple pairings of the CS and US (Hunt and Amit, 1986). Third, in most types of classical conditioning when the CS is presented repeatedly without the US, the CR weakens and then

disappears over time through a process called “habituation”. In CTA, the CR persists for much longer and may never disappear completely (Hunt and Amit, 1986).

The differences between CTA and “typical” classical conditioning can be explained through the evolutionary function of CTA. Most animals in the wild frequently encounter toxic or rotten food that can be extremely dangerous (Hunt and Amit, 1986). An animal that eats toxic food often will not become sick until hours or days later. Animals that can learn to associate the toxic food with sickness after only one pairing, even if a long period of time passes between the food and sickness, and then avoid that food for a long period of time or even for the rest of their lives should have an evolutionary advantage over animals that repeatedly eat toxic food (Hunt and Amit, 1986).

Although most of the differences between CTA and typical classical conditioning have been adequately explained, one of CTA’s most unique features still is not well understood: no matter what type of drug is used, the animal typically shows an aversion rather than a preference (Hunt and Amit, 1986). Drugs with rewarding effects, including methamphetamines, cocaine and opioids, which normally produce conditioned preference, produce conditioned aversion in CTA (Parker, 1995; Reicher and Holman, 1977; Wise et al., 1976).

There are several theories as to why aversion is the norm when tastes are paired with addictive drugs. Hunt and Amit (1986) speculated that every drug causes a mix of positive and negative effects and that, for evolutionary reasons, animals are more sensitive to the negative effects when drugs are paired with a taste. Hunt and Amit’s theory would explain why CTA and CPP can often be observed in the same animal (White et al., 1977; Wise et al., 1976). Their theory would also explain apparent behavioral and neurological differences between CTA

induced by emetic drugs and CTA induced by drugs that also show CPP (Mucha and Hertz, 1984; Parker, 1995)

If it were somehow possible to disentangle the negative effects from the positive effects, or to give the positive effects increased salience, then it would be theoretically possible to show Conditioned Taste Preference. In one study, researchers were apparently able to do just that. Mucha and Hertz (1984) found that, while high doses of morphine, fentanyl and sufentanil produced CTA, low doses of the same drugs produced CTP. The researchers found that at high doses, kappa opioid receptors, which are responsible for the aversive aspects of opioid use, were activated (Bechara and Van der Kooy, 1987; Land et al., 2009; Mucha and Hertz, 1984; Shippenberg and Herz, 1986). At low doses, only mu receptors, which are responsible for the positive aspects of opioids, were activated (Land et al., 2009; Mucha and Hertz, 1984; Shippenberg and Herz, 1986). The results suggest that simply using a low dose of opioids may be enough to avoid activation of the aversive kappa receptors and CTA.

If Mucha and Hertz's results were replicated, or if another method can be found to make the positive, but not aversive, aspects of drugs more salient when paired with tastes, CTP could feasibly serve as another classical conditioning model to study drug abuse. This model could have many of the same advantages of CPP. In addition, it would allow researchers to study the discrete cues involved in drug conditioning, create dose-effect curves by pairing multiple different drugs doses with different tastes, and would eliminate the forced choice confound found in CPP. However, the high probability of causing CTA instead of CTP could seriously limit the model. In order for a CTP model to be feasible, a reliable way to induce CTP without inducing CTA must be found.

One avenue to avoid CTA could lie in the distinction between delay and trace conditioning. Delay conditioning is the most commonly used type of conditioning in lab settings. During delay conditioning, there is a period of overlap between the presentation of the CS and the presentation of the US (Solomon and Groccia-Ellison, 1996). For example, in CPP the animal begins to experience the effect of the drug it has been given (US) while still in the CPP chamber (CS). In contrast, trace conditioning occurs when there is a period where no stimulus is present in between the presentation of the CS and the US (Solomon and Groccia-Ellison, 1996). For example, if an animal is shocked (US) thirty seconds after a tone stops playing (CS).

This distinction between delay and trace conditioning could be key to understanding why previous studies have almost always found taste aversion. As Hunt and Amit (1986) noted, most addictive drugs have both rewarding and aversive effects. Rewarding effects generally occur earlier in the drug's time course (Harris and Gewirtz, 2004; Rothwell et al., 2009). Aversive effects, which can include symptoms such as anxiety and depression, generally occur later and are collectively known as "withdrawal" (Harris and Gewirtz, 2004; Rothwell et al., 2009).

We hypothesize that animals always show CTA and not CTP because of trace conditioning due to drug withdrawal. Animals may learn to associate the aversive stimulus of drug withdrawal with the conditioned taste paradigm through trace conditioning and, in response, learn to avoid the food they associate with withdrawal. Although delay conditioning generally has a stronger effect than trace conditioning, the unique properties of the CTA paradigm suggest that animals may be evolutionarily predisposed to quickly and strongly associate illness with the taste of food or water (Solomon and Groccia-Ellison, 1996). The ability to associate illness with food even after a long interval would be especially evolutionarily advantageous because

foodborne illness occurs hours or days after the initial exposure (Glynn and Palmer, 1992; Granum and Lund, 1997; Riedo et al., 1994).

If aversive trace conditioning is unusually strong in the taste aversion paradigm, then it may overpower any drug preference caused by delay conditioning. However, if withdrawal could be delayed for long enough that it no longer caused trace conditioning, then it might be possible to demonstrate CTP from the initial rewarding effects of a drug.

The Present Research

The purpose of the present research was to determine whether conditioned odor preference and conditioned taste preference are feasible models for drug use. More specifically, we attempted to demonstrate that mice can be classically conditioned to associate morphine with both odor and taste. Our hypothesis was that both odor and taste conditioning paradigms would be connected to mice's preference for drug-paired cues and hence would be proven valuable as novel tools for evaluating drug addiction tendencies in mice.

The research included experiments on odor and taste conditioning. In the first experiment, mice were exposed to two odors, one of which was paired with morphine and one of which was paired with saline. We predicted that during the testing phase, the animals would spend more time investigating the morphine-paired odor than the saline-paired odor.

In the second and third experiments, mice were exposed to two different flavored food types, one of which was paired with morphine and one of which was paired with saline. We predicted that in the second experiment, which was designed to act as a control, mice would demonstrate taste aversion consistent with what has been shown in previous literature. In the third experiment, mice also received a second injection of either morphine or saline before they

would have begun to enter withdrawal. We hypothesized that this second injection would delay withdrawal long enough that the mouse would not learn to associate the negative effects of withdrawal with the food and would therefore develop a preference, rather than an aversion, for the taste. We predicted that the mice in the third experiment would eat more of the morphine-paired food than the saline-paired food during the testing phase.

Non-CPP classical conditioning models are promising research paradigms that have been almost completely ignored by current drug addiction researchers. Drug addiction is arguably the most pressing public health emergency facing the United States today. In order to combat drug addiction, it is important to fully utilize every possible research avenue. If validated and more fully developed, new classical conditioning models have the potential to become a promising new tool for researchers involved in the vital task of understanding and eliminating drug addiction.

Methods

Experiment One: Conditioned Odor Preference

The purpose of Experiment One was to determine whether odor can be paired with morphine through a standard classical conditioning procedure. Mounting putty was used to mount unscented cotton swabs to opposite walls of a cage using the procedure in Yang and Crawley (2009). Twenty C57/B6 mice were allowed to habituate to the cage through daily, 30 minute sessions for three to five days.

After the completion of the habituation trials, one cotton swab was scented using a dilution of almond extract and one cotton swab was scented using a dilution of vanilla extract. During the pretest, mice were allowed to explore the cage and investigate both cotton swabs for

30 minutes. After the pretest, mice experienced six days of conditioning. During conditioning, both cotton swabs in the cage were scented with either vanilla or almond extract. Mice were injected with 5 mg/kg saline/morphine on alternate days and allowed to explore the cage for 30 minutes. For half of animals, the morphine was paired with vanilla scent and the saline was paired with almond scent. For the remaining animals, the opposite procedure was followed. After conditioning, the animals completed a final posttest that was identical to the pretest (see Figure 1 for summary of protocol). The pretests and posttests were video recorded. The recordings were scored by an observer blinded to the identity of the odors to determine how much time the mice spent investigating each cotton swab. Intra-rater reliability was determined using a Pearson Correlation. The results were analyzed using paired sample t-tests.

Experiment Two: Conditioned Taste Aversion

Experiment Two was a pilot study designed to confirm previous studies' findings on taste aversion. Before the experiment began, five C57/B6 mice were placed on a restricted feeding schedule over the course of a week. On the first day, food was available for the mice to eat between 9:00AM and 6:00PM. During each subsequent day, the window during which food was available was shortened by one to two hours. At the end of the week, food was available for only three hours a day, during which time the mice had to consume all of their daily calories. This three-hour restricted feeding schedule was maintained for the duration of the experiment. Throughout the experiment mice were monitored to ensure they maintained a minimum of 85% of their free-feeding weight.

Two dishes containing food pellets were placed on opposite sides of a cage. Mice were allowed to habituate to the cage through two 30-minute sessions performed on consecutive days. During these habituation sessions the bowls were filled with unflavored food pellets. A pretest

was performed after the completion of the habituation days. During the pretest, mice were placed in the same cage as was used during habituation for 30 minutes. However, one dish was filled with chocolate-flavored pellets and one dish was filled with banana-flavored pellets. The dishes were weighed before and after the test to determine how much food the mice consumed. During conditioning, both dishes were filled with either chocolate-flavored or banana-flavored food pellets. Mice were injected with 5-mg/kg saline/morphine on alternate days and allowed to explore the cage for 30 minutes. Because animals showed an initial preference for the chocolate pellets during the pretest, we used a biased design in which morphine was paired with the chocolate pellets for all the animals. After conditioning, the mice completed a final posttest that was identical to the pretest (see Figure 1 for summary of protocol). The results were analyzed using a two-way repeated measures Analysis of Variance (ANOVA) and a Bonferroni post hoc test.

Experiment Three: Conditioned Taste Preference

The purpose of Experiment Three was to determine whether mice that are given an additional injection of morphine to delay withdrawal will develop a preference for flavored food paired with morphine. Five C57/B6 mice were placed on a restricted feeding schedule following the same procedure as Experiment Two. Two dishes containing food pellets were placed in the center of a cage. Mice were allowed to habituate to the cage through two 30-minute sessions performed on consecutive days. During the habituation sessions, both the bowls were filled with marshmallow-flavored food pellets.

Because a different brand of food pellets were used during Experiment Three than during Experiment Two, three pretests were performed on consecutive days in order to determine for which combination of flavors mice showed the smallest initial difference in preference. During

pretests, mice were placed in the same cage as was used during habituation for 30 minutes. Animals were injected with 5-mg/kg saline before the pretest and again 2.5 hours later. During the first pretest, one bowl was filled with piña colada-flavored food pellets and one bowl was filled with berry-flavored food pellets. During the second pretest, one bowl was filled with chocolate-flavored food pellets and one bowl was filled with banana-flavored food pellets. During the third pretest, one dish was filled with piña colada-flavored pellets and one dish was filled with banana-flavored pellets. The dishes were weighed before and after the test to determine how much food the mice consumed. Because mice showed the least initial difference in preference between chocolate and banana, those two flavors were chosen for use during condition.

During conditioning, both dishes were filled with either chocolate-flavored or banana-flavored food pellets. Mice were injected with 5-mg/kg saline/morphine on alternate days and allowed to explore the cage for 30 minutes. 2.5 hours after the first injection, mice received an additional injection of 5-mg/kg morphine/saline in order to delay withdrawal. Because animals showed a slight preference for the banana pellets during the pretest, morphine was paired with the banana pellets for all the animals. After conditioning, the mice completed a final posttest that was identical to the banana/chocolate pretest (see Figure 1 for summary of protocol). The results were analyzed using a two-way repeated measures ANOVA and a Bonferroni post hoc test.

Results and Discussion

Experiment One: Conditioned Odor Preference

Conditioned Place Preference (CPP) is one of the most commonly used models to study drug addiction. CPP is based on the principles of classical conditioning and uses place as a

conditioned stimulus. Despite its popularity, CPP has several limitations. These limitations could be eliminated by new models that use discrete stimuli as the conditioned stimulus. The first step in proving the feasibility of new models is to demonstrate that classical conditioning can be used to pair a discrete stimulus with a drug. The first discrete stimulus we examined was odor. We hypothesized that mice would show a preference for an odor that had been repeatedly paired with morphine.

In order to test the hypothesis, morphine was repeatedly paired with either almond or vanilla odor, while on alternate days saline was paired with the other odor as a control. Before and after conditioning, mice were given a test in which they were allowed to investigate both odors. Investigation time for the two odors during the pretests and posttests was measured by a blind rater. Intra-rater reliability was determined using a Pearson correlation ($r = .864$).

If mice showed a preference for one odor before conditioning began, as measured by significant differences in investigation time, the results of the study could be confounded. To control for this possibility, a paired samples t-test was performed to compare investigation time for the two scents during the pretest. No significant differences were found ($p = .847$; see Table 2 and Figure 2 for complete results). Paired samples t-tests were used to determine whether there were differences in investigation time between the pretest and posttest for the morphine-paired scents or between the morphine and saline-paired scents during the posttest. If mice did develop a preference for the morphine-paired odor, they would be expected to spend significantly more time investigating the morphine-paired odor during the posttest than during the pretest and to spend more time investigating the morphine-paired odor than the saline-paired odor during the posttest. In contrast, a t-test showed that mice in the condition where almond was the experimental odor spent significantly more time investigating the almond scent during the pretest

than during the posttest (p = .036). No other results were significant. The results were not consistent with the hypothesis that mice would develop a preference for the morphine-paired odor.

Experiment Two: Conditioned Taste Aversion

Like odor, taste is a discrete stimulus that could potentially be used as a conditioned stimulus in drug addiction models. The purpose of this experiment was to determine whether, as has been reported in previous literature, mice show an aversion for a flavored food that has been repeatedly paired with morphine (Parker, 1995; Reicher and Holman, 1977; Wise et al., 1976). In order to replicate previous research, tests were performed before and after a conditioning phase in which chocolate and banana-flavored food were paired with morphine and saline, respectively. During the pretest and posttest, mice were allowed to choose between both flavors and the amount of each type of food eaten was measured.

If mice preferred banana-flavored food to chocolate-flavored food before conditioning, then this pre-existing preference could be a confound. A paired samples t-test showed that during the pretest, mice in fact ate significantly more chocolate-flavored (M = 376.88 mg, SD = 104.15) than banana-flavored (M = 58.16 mg, SD = 90.24) food, t(4) = 4.0253, p = .0158 (see Figure 3 for summary of results). Because of this large initial preference, we chose to use a biased design in which the chocolate-flavored food was paired with morphine for all of the animals. The purpose of the biased design was to limit the amount of confounding due to differences in initial preference.

The main purpose of the experiment was to determine whether mice develop a taste aversion for a flavor paired with morphine. If mice did develop an aversion to the morphine-

paired flavor, then an interaction between flavor and test would be expected. In order to test for an interaction, a two-way repeated measures ANOVA test was performed to compare food consumption based on flavor (banana vs. chocolate) and test (pretest vs. posttest). Simple main effects analysis showed that the animals ate significantly more food during the posttest than during the pretest (p <.001) and ate significantly more banana-flavored than chocolate-flavored food (p <.001). As expected, there was a statistically significant interaction between the effects of pre/posttest and flavor, $F(1, 4) = 114.348$, $p < .001$.

If the mice developed a taste aversion, then they would also be expected to eat less chocolate-flavored food during the posttest than during the pretest and less chocolate-flavored food than banana-flavored food during the posttest. In order to test for these effects, a Bonferroni post hoc test was performed. Consistent with the hypothesis that mice would form an aversion to a taste paired with morphine, the Bonferroni test found that during the posttest mice ate significantly less chocolate-flavored (M = 53.36 mg, SD = 102.51) than banana-flavored (M = 1225.98 mg, SD = 189.28) food (p =.005). Also consistent with the hypothesis, the mice ate less chocolate-flavored food during the posttest (M = 53.36 mg, SD = 90.24) than during the pretest (M = 376.88 mg, SD = 104.15), although the result was not significant (p = .065).

Experiment Three: Conditioned Taste Preference

We hypothesized that the taste aversion reported in previous literature comes about because animals learn to associate aversive withdrawal symptoms with taste through trace conditioning. Therefore, delaying withdrawal until after the window for trace conditioning may eliminate taste aversion. If trace conditioning were eliminated, animals might even demonstrate a taste preference because they would learn to associate the rewarding aspects of the drug with the taste through delay conditioning. In order to test this hypothesis, we repeated the procedure used

in Experiment Two, but during the pretest and conditioning phases gave the animals an additional injection of saline/morphine 2.5 hours after the initial injection in order to delay withdrawal.

If mice preferred one flavor of food before conditioning, then this pre-existing preference could be a confound. A paired samples t-test showed that during the pretest, there were no significant differences in the amount eaten of each flavor, $t(4) = 1.0837$, $p = .3394$ (see Figure 4 for summary of results). Even though the difference was not significant, mice did eat more of the banana-flavored food (M = 383.42, SD = 258.99) than of the chocolate-flavored food (M = 135.20, SD = 278.84).

The main purpose of the experiment was to determine whether the mice developed an aversion, preference, or neither to the morphine-paired flavor. If mice did not develop an aversion, then no interaction between flavor and test would be expected. In order to test for an interaction, a two-way repeated measures ANOVA test was performed to compare food consumption based on flavor (banana vs. chocolate) and test (pretest vs. posttest). Simple main effects analysis showed no significant difference in the amount of food eaten during the pretest vs. posttest ($p = .896$) or in the amount of each flavor of food eaten ($p = .290$). As expected, there was not a statistically significant interaction between the effects of pre/posttest and flavor, $F(1, 4) = .046$, $p = .840$.

If we were successful in eliminating taste aversion, mice would be expected to eat an equal amount of banana and chocolate-flavored food, or even to show a preference for the banana-flavored food. A Bonferroni post hoc test was performed to compare chocolate and banana-flavored food consumption during the posttest and banana-flavored food consumption during the pretest and posttest. Consistent with the hypothesis that mice would not form an

aversion to a taste paired with morphine, the Bonferroni test found no significant differences. Although the difference was not significant, mice did eat more banana-flavored (M = 419.64, SD = 453.78) than chocolate-flavored (M = 121.94, SD = 205.70) food during the posttest (p = 1.0). Mice also ate more banana-flavored food during the posttest (M = 419.64, SD = 453.78) than during the pretest (M = 383.42, SD = 258.99; p = 1.0). Both of these results are consistent with the hypothesis that mice would develop a preference for a flavor paired with morphine.

General Discussion

Our goal was to determine whether mice can be conditioned to develop a preference for either odors or tastes when they are paired with morphine. Our results suggest that mice can be conditioned to associate taste, but not odor, with morphine. In the taste preference experiment, mice did not spend any more time investigating the morphine-paired odor than the saline-paired odor and actually spent less time investigating the morphine-paired odor after conditioning than before conditioning, although the difference was only significant for one of the experimental groups. In our taste aversion experiment, mice ate significantly less of the flavored food paired with morphine after conditioning than before conditioning. After conditioning the mice also ate significantly less of the morphine-paired flavor than the control saline-paired flavor. In our taste preference experiment, mice ate more of the morphine-paired flavor after conditioning then before conditioning and ate more of the morphine-paired food than the saline-paired food after conditioning, although neither effect was significant. Our results suggest that it may be possible to eliminate taste aversion and, much more tentatively, to demonstrate taste preference by delaying withdrawal.

Conditioned Odor Preference

The inability to create a conditioned odor preference was, we believe, one of our most surprising results. Odor stimuli are extremely salient to mice and play an important role in how they interact with their environment (Apfelbach, 2005; Gilder and Slater, 1978; Yang and Crowley, 2009). As such, odor should be relatively easy to condition in mice as compared to other potential stimuli. In fact, a large body of evidence has shown that it is possible to pair odor with a variety of unconditioned stimuli, including rewarding drugs (Bouslama et al., 2005; Goddyn et al., 2008; Heyser et al., 1990; Johanson and Teicher, 1980; Svyf, 2014).

It is possible that the results of our experiment were inconsistent with those of previous research because investigation time is a uniquely poor measure of conditioning. None of the other studies that we encountered used investigation time as a measure of conditioning. Instead, they used measures such as startle response or time spent in a chamber that contained the odor (Goddyn et al., 2008; Heyser et al., 1990). These measures may not suffer from the same limitations that we encountered with investigation time.

One of the largest limitations of using investigation time was the relatively small amount of time that mice spent investigating the odors as opposed to engaging in other activities. It was hoped that allowing animals to engage in activities not related to odor investigation would be one of the model's strengths. CPP forces animals to choose between the control and experimental chambers, which can make it difficult to determine if they actually prefer the experimental chamber or are simply avoiding the control chamber because they have learned to associate it

with withdrawal. In contrast, the conditioned odor test did not force animals to choose between the two odors because they could choose not to investigate either one.

While a good idea in theory, in reality the lack of forced choice meant that out of each thirty minute session, mice on average spent a total of only a few seconds investigating the source of the odors. The small amount of investigation time made the videos difficult to score accurately. It also meant that even seemingly small differences in investigation time between animals were important statistically. For example, even if an animal only spent six seconds out of thirty minutes investigating the odors, that might be three or even six times as much investigation time as another animal. The high amount of variation between animals made it difficult to find statistically significant results and, by extension, to reach any firm conclusions about the experiment.

A second problem with our experimental measure is that it may have been seriously confounded by novelty. The pretest was the first time that the mice were exposed to either odor. In contrast, by the time of the posttest, the mice had been exposed to both odors multiple times during both the pretest and conditioning. This means that the odors were relatively novel during the pretest and relatively familiar during the posttest. A large body of previous research has showed that animals generally spend more time investigating a novel stimulus as compared to a familiar stimulus (Fantz, 1964; Williams, 1963). The results of the research showed that mice spent more time investigating the experimental odor during the pretest than during the posttest, although the difference was only significant in one group. The decreased amount of investigation during the posttest could be because of the difference in stimulus novelty between the two tests. It is even possible that mice did develop a preference for the odor that was paired with morphine,

but that the preference was obscured by a stronger effect in the opposite direction caused by novelty.

Our results suggest that if Conditioned Odor Preference is to become a viable model for drug addiction, an alternative to investigation time must be found to measure preference. Previous researchers have found success measuring the amount of time animals spend in a chamber with the experimental odor. This would eliminate two of the limitations of investigation time: the difficulty of scoring videos accurately and the overall small amount of investigation time. However, it would also reintroduce the forced choice confound found in standard CPP. Future researchers may be able to develop another measure for preference that does not eliminate the advantages of CPP. However, with current preference measures, it is likely that even if researchers could demonstrate a conditioned preference for an odor paired with morphine, the model would have too many limitations to make Conditioned Odor Preference an acceptable replacement for CPP.

Conditioned Taste Aversion

In contrast with the results of the odor conditioning experiment, the results of the taste aversion experiment were largely consistent with previous research. A large number of previous experiments have all shown that mice develop an aversion to flavored food paired with drugs that are generally considered rewarding such as morphine, although the exact mechanism remains uncertain (Hunt and Amit, 1986; Parker, 1995; Reicher and Holman, 1977; Wise et al., 1976). The present taste aversion experiment was performed only as a control to compare with the taste preference experiment. As expected, the mice showed a strong aversion to the flavor that was paired with morphine.

The taste aversion did have one unexpected result: although the amount of the morphine-paired food eaten decreased from the pretest to the posttest, the total amount of food increased because animals greatly increased their consumption of the saline-paired food. Because it was being used as a control, the experiment should not have increased preference for the saline-paired food and the total amount of food eaten should have remained relatively constant.

Sanger and McCarthy (1980) found that morphine increases the food intake of free-feeding rats, but reduces the food intake of food-deprived rats. The authors hypothesized that the change in food consumption may have been related to the role of the opioid system in regulation of nutrient acquisition and energy expenditure. The mice used in the present research were food deprived for the duration of the experiment and, consistent with Sanger and McCarthy, showed greatly decreased food consumption during conditioning days when they were exposed to morphine (see Figure 3). Possibly as a result, the weight of the animals decreased throughout the experiment and by the time of the posttest, all five animals were below 85% of their original free-feeding weight. It is possible that the increased food consumption during the posttest was seen because the mice were compensating for the calorie deficit that they had acquired over the course of the experiment.

More research is needed to determine what effect morphine has on food consumption and whether that effect differs under different circumstances. Researchers should also explore the specific biological mechanisms that allow morphine to influence food consumption and why those mechanisms would lead to increased food consumption under some circumstances but decreased food consumption under different circumstances. Understanding the link between morphine and food consumption is especially important because increased/decreased food

consumption due to the biological effects of morphine has the potential to confound any future experiments performed on taste preference/aversion.

Conditioned Taste Preference

Although our control experiment clearly showed taste aversion, our final taste experiment did not have any significant differences. The fact that there were no significant differences suggests that taste aversion was successfully eliminated by giving the animals a second injection of morphine to delay withdrawal. The finding contradicts previous research, which has overwhelmingly found that pairing rewarding drugs with flavored food or water causes taste aversion (Parker, 1995; Reicher and Holman, 1977; Wise et al., 1976). The finding is consistent with our hypothesis that taste aversion is caused by trace conditioning and can be eliminated by delaying withdrawal so that it occurs outside the window of time in which trace conditioning is possible.

We also hypothesized that if trace conditioning was eliminated, then delay conditioning from morphine's rewarding effects may be unmasked. If this were the case, we would expect to observe taste preference. Mice did eat more of the experimental flavor than the control flavor during the posttest and ate more of the experimental flavor during the posttest than the pretest, but neither effect was significant. It is very possible that the failure of either effect to reach the level of significance could indicate that the correlation was illusory and simply due to chance. However, it is also possible that the effect indicates that the experiment was successful in unmasking taste preference.

The latter interpretation is supported by differences between the conditioning trials for Experiment Two, when the mice were not given an additional morphine injection, and

Experiment Three, when the mice were given an additional injection (see Figures 3 and 4). During Experiment Two, the amount of food eaten decreased on each consecutive morphine day, and the mice ate less morphine-paired food during the posttest than on any other day. Conversely, in Experiment Three the amount of food eaten dropped sharply from the pretest to the first experimental day of conditioning, but then increased on each consecutive day of conditioning. The mice ate more morphine-paired food during the posttest than on any other day of the experiment.

One interpretation of these differences could be that in Experiment Two, the association between withdrawal symptoms and the flavor of food was reinforced every experimental day. As the association grew stronger, the mice ate less and less food. In Experiment Three, the mice may have eaten less food on the first experimental conditioning day either because of morphine's effect of decreasing food consumption, as was previously discussed, or because the animals were initially wary of the food because it was accompanied by novel physiological effects. As the experiment progressed, the animals lost their initial wariness to morphine's physiological effects and began to associate morphine's rewarding effects with the flavored food. This association grew stronger with each repeated exposure to morphine paired with the experimental flavor, which caused the mice to eat more food on each consecutive day. It is possible that if the experiment had more conditioning days, the association would have become strong enough to cause a significant effect.

It is also possible that the effect was insignificant because withdrawal was not delayed long enough to completely eliminate aversive learning and that the resulting decrease food consumption masked most of the preference-induced increase in food consumption. If this were the case, then future studies may be able to demonstrate a stronger taste preference by

administering the first morphine injection an hour after exposure to the experimental flavor and the second morphine injection two to three hours after the first. This would delay withdrawal by an additional one to two hours and potentially unmask a taste preference. Finally, we may not have found a significant preference because the experiment, which only included five animals, did not have enough power.

Although the experiment's results appear promising, it is important to be cautious about drawing strong conclusions. Not only were the results insignificant, but the experiment also suffered from several limitations. First, the type of food used in Experiment Three was different than in Experiment Two, which limits Experiment Two's efficacy as a control and makes it difficult to draw direct comparisons between the two experiments. It is possible that any differences seen between the two experiments were due to differences in the animals' preference for one type of food over the other.

Second, the mice in both experiments became underweight over the course of the study. The animals' low weight was a confound in of itself because it could have affected the animals' eating behavior. Additionally, the mice in Experiment Three, but not in Experiment Two, were left with extra food overnight after the final day of conditioning in an attempt to increase their weight. The food was regular, unflavored chow, meaning that the mice were not given any extra exposure to the experimental or control food. Additionally, the pattern of increasing food intake on each consecutive morphine day was observed before the mice received extra food, suggesting the pattern was not caused entirely by the change in feeding schedule. Despite these mitigating factors, the change in protocol still introduced an additional uncontrolled variable and it is impossible to say with certainty how it may have affected the results.

Future Directions

Experiments Two and Three were designed to serve as a pilot study, rather than to stand on their own, and should be viewed in that light. While the results of the study are extremely preliminary, they do justify performing further research. We plan to replicate the present research with a better-controlled experiment and with more animals. In future experiments, we plan to eliminate differences between the experimental and control trials, such as type of food, which should make any analysis more clear-cut.

If future experiments are able to replicate the results of this study, then it may suggest several important avenues for researchers to follow. From a theoretical standpoint, it is important to perform further research to either confirm or deny our hypothesis that taste aversion is caused by trace conditioning. If taste aversion can be eliminated by delaying withdrawal with multiple morphine injections, it strongly suggests that taste aversion is caused by withdrawal symptoms.

However, there are other possible explanations. For example, previous research has established that morphine can impair learning (Castellano 1975; Ukai and Lin 2002). Therefore, instead of preventing taste aversion specifically, the second injection of morphine may simply have impaired learning of any kind. Future research could test this hypothesis by administering a non-opioid drug that blocks withdrawal, such as clonidine or propranolol, rather than a second injection of morphine (Rothwell et al., 2009). If animals did not develop taste aversion using this protocol, it would provide further evidence that withdrawal is responsible for taste aversion.

Another possibility is that increased exposure to morphine from receiving multiple injections may allow animals to become more accustomed to its effects, both positive and negative, and to adjust their eating behavior accordingly. No matter what explanation is

ultimately reached, determining why repeated morphine injections eliminate taste aversion is important to better understand the theoretical underpinnings of taste aversion and why it differs so dramatically from other forms of classical conditioning.

From a more practical standpoint, future research will also be important in establishing Conditioned Taste Preference's efficacy as a complement to CPP. Studies may show that repeat drug injections eliminate taste aversion, but do not cause taste preference. If this is the case, then the efficacy of using taste as a discrete stimulus to study drug use may be extremely limited. Although research on the aversive properties of drugs is important, it is at least equally as important to study the rewarding aspects of drug abuse so that researchers can better understand why addiction occurs and how to prevent it.

If, on the other hand, research reveals that repeat drug injections do cause taste preference, then there is a much stronger possibility that taste could be used successfully to study drug abuse. If future experiments show taste preference, then researchers should focus on further developing Conditioned Taste Preference as an animal model for drug use. It is especially important to study Conditioned Taste Preference's advantages and limitations. If we are correct that Conditioned Taste Preference has all or most of the same advantages as CPP but has fewer limitations, then it could prove to be a valuable tool for researchers to use in addition to or in place of CPP.

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Conditioning type	Name	Measure of reward and/or aversion	Advantages					Limitations					
			Mimics real-world drug use	Fast, low cost and easy to use	Measures reward and aversion	S-shaped dose-effect curve	Can measure stimulus interactions	Requires Surgery	Confounds from tolerance/sensitization	Novelty confound	Initial preference for "neutral" stimulus	Multiple test groups for dose-effect curves	Confounds from Forced choice
Operant Conditioning	Self-Administration	Maximum lever presses for a dose of the drug	Yes					Yes	Yes				
Classical Conditioning	Conditioned Place Preference (CPP)	Time spent in drug-paired chamber		Yes	Yes	Yes				Yes	Yes	Yes	Yes
	Conditioned Object Preference (COP)	Time spent investigating drug-paired object		Yes	Yes	Yes	Yes			Yes	Yes		
	Conditioned Odor Preference	Time spent investigating drug-paired odor		Yes	Yes	Yes	Yes			Yes	Yes		
	Conditioned Taste Preference	Amount of drug-paired flavored food eaten		Yes	*	Yes	Yes			Yes	Yes		

Table 1: Animal models for drug addiction. Animal models that are currently in use or could potentially be used to study drug addiction. Includes information on the type of conditioning (operant or classical) that each model is based on, how reward/aversion is measured in each model, and each model's advantages/limitations.

*Most studies that have paired a drug to flavored food/water have only been able to measure aversion. One of the purposes of this study is to demonstrate that it is possible to measure reward using a taste preference task.

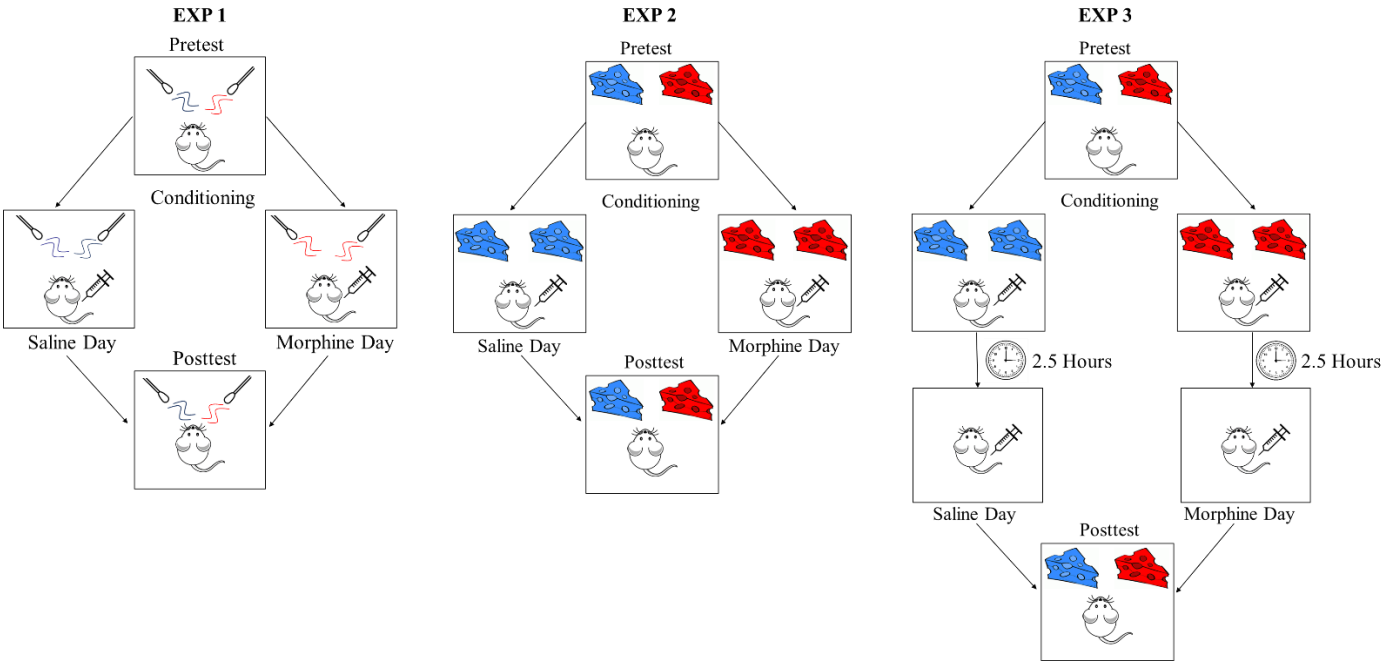

Figure 1: Schematic for experimental methods. EXP 1: Mice were allowed to investigate two odors during the pretest. During conditioning, the mice were injected with saline and exposed to the control odor or injected with morphine and exposed to the experimental odor on alternating days. During the posttest, mice were again allowed to investigate the two odors. EXP 2: Followed the same procedure as EXP 1, but used flavored food instead of odors. EXP 3: Followed the same procedure as EXP 3, but during conditioning mice were given an additional injection of saline/morphine 2.5 hours after the first injection.

Scent paired with morphine	T-Test	*p*-value
None (Control)	almond pretest vs. vanilla pretest	.847
Almond	almond posttest vs. vanilla posttest	.202
	almond pretest vs. almond posttest	.036*
Vanilla	almond posttest vs. vanilla posttest	.23
	vanilla pretest vs. vanilla posttest	.599

Table 2: Conditioned Odor Preference Results. Results of T-tests performed to compare the time spent investigating each scent in the pretest, posttest and pretest vs. posttest for mice in both the almond/morphine and vanilla/morphine conditions. N = 20 mice.

*Significant at $p < .05$

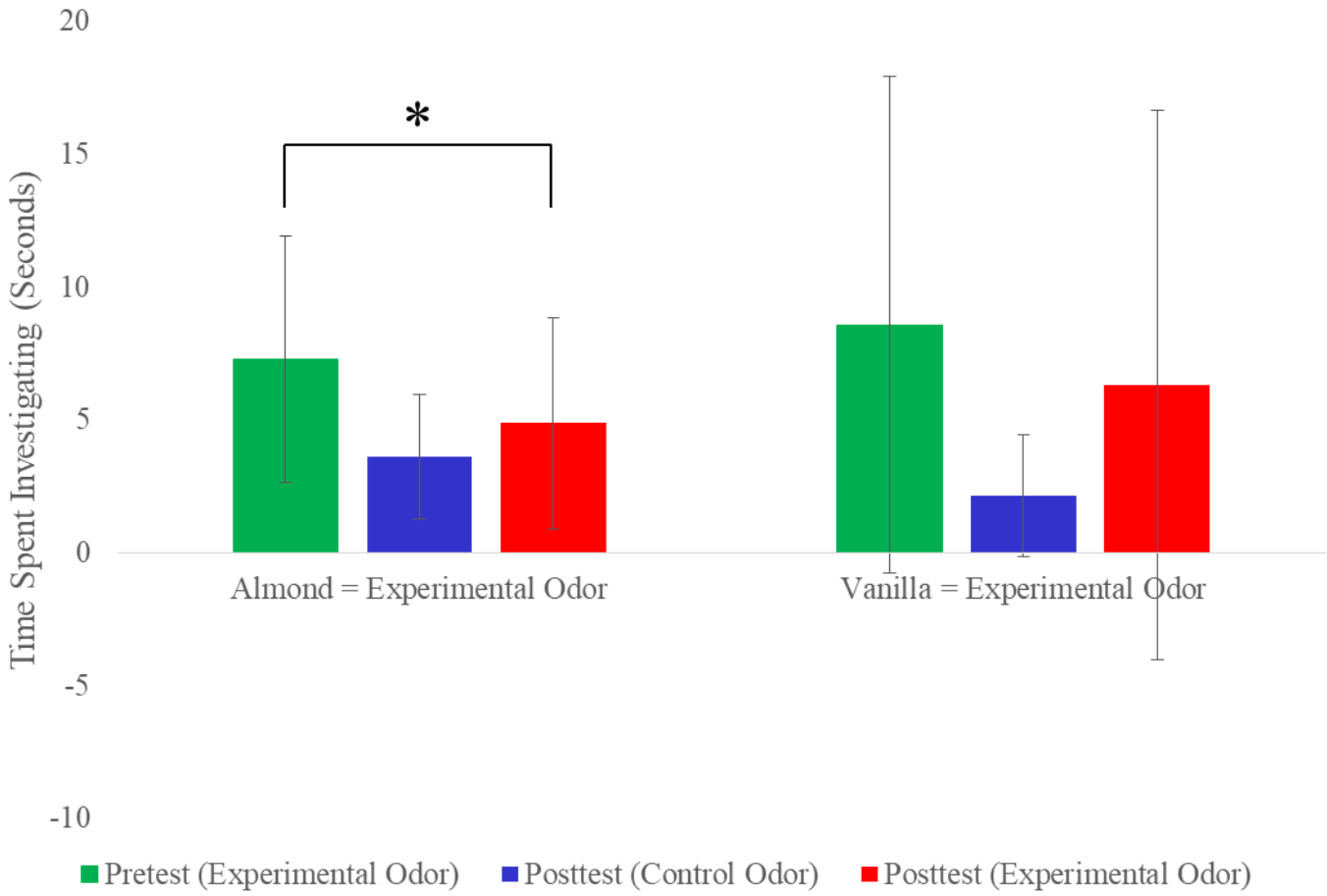

Figure 2: Conditioned Odor Preference Results. Time spent investigating (in seconds) experimental and control odors during the pretest and posttest in Experiment One. The experimental odor was the odor paired with morphine. The control odor was the odor paired with saline. When almond was the experimental odor, vanilla was the control odor and vice versa. Error bars represent standard deviation. N = 20 mice.

*Significant at $p < .05$

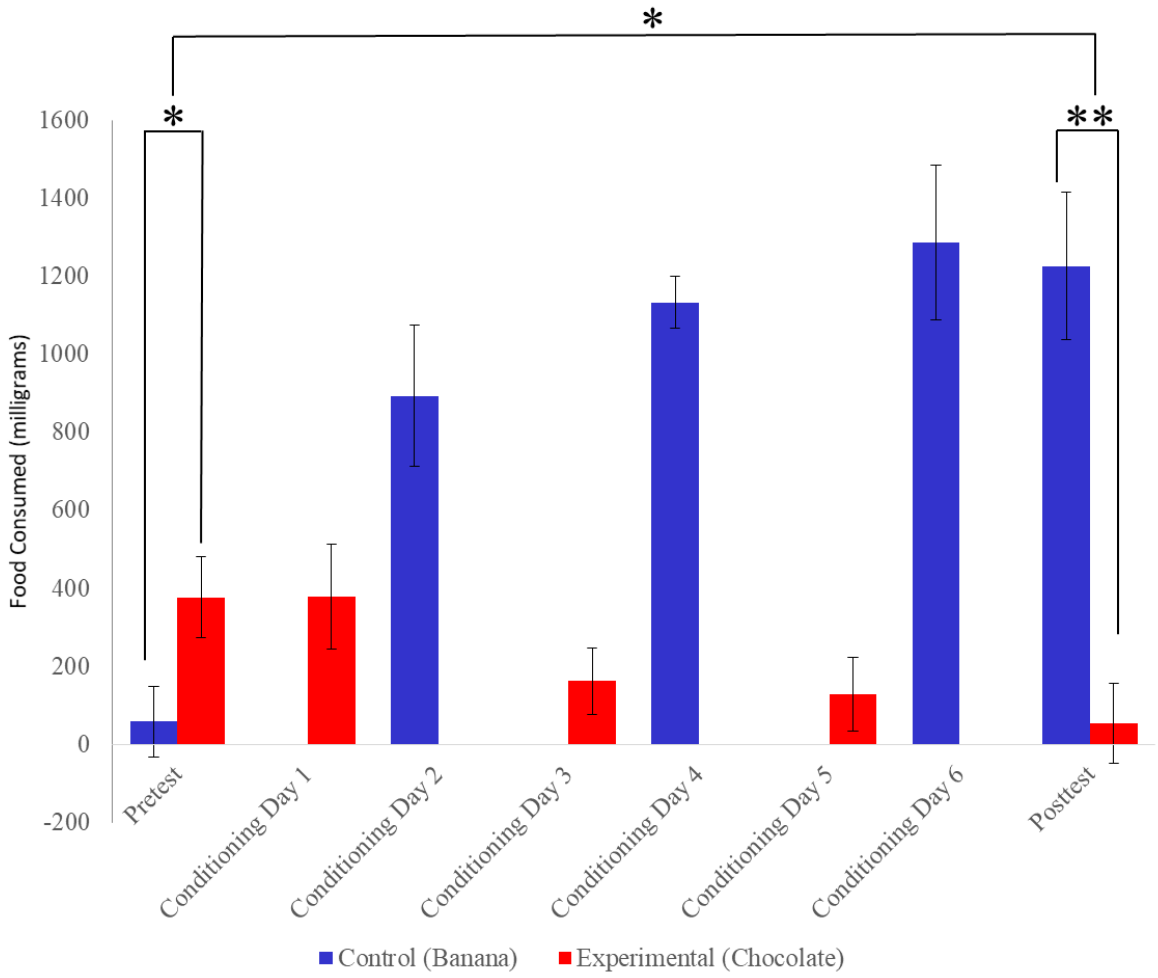

Figure 3: Conditioned Taste Aversion Results. Amount of control and experimental foods consumed (in milligrams) during the pretest, conditioning and posttest in Experiment Two. Error bars represent standard deviation. Note: mice were only exposed to one flavor of food (experimental or control) on each day of conditioning. N = 5 mice.

*Significant at $p < .05$

**Significant at $p < .01$

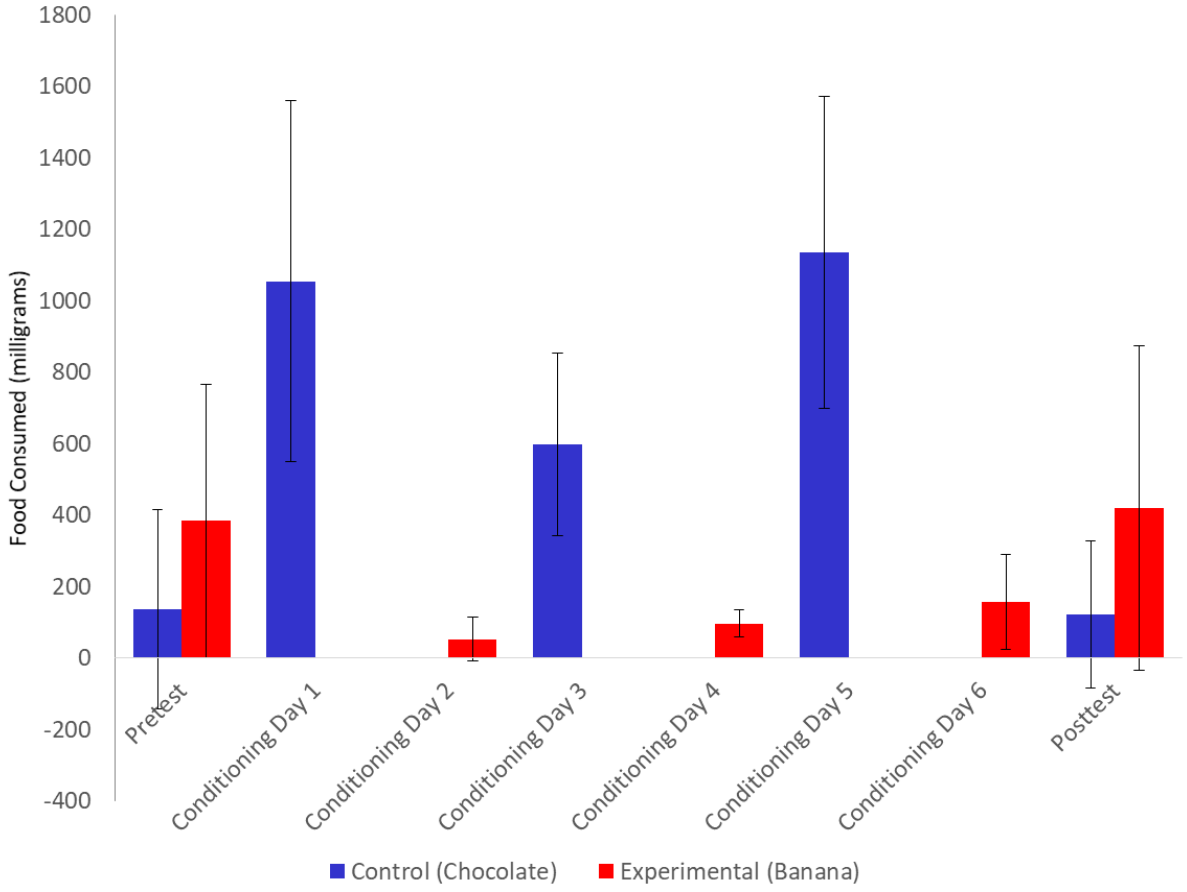

Figure 4: Conditioned Taste Preference Results. Amount of control and experimental foods consumed (in milligrams) during the pretest, conditioning and posttest in Experiment Three. Error bars represent standard deviation. Note: mice were only exposed to one flavor of food (experimental or control) on each day of conditioning. N = 5 mice. No significant results were found.